

## BACTERIOLOGY

# Cystic fibrosis–adapted *Pseudomonas aeruginosa* quorum sensing *lasR* mutants cause hyperinflammatory responses

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Cystic fibrosis lung disease is characterized by chronic airway infections with the opportunistic pathogen *Pseudomonas aeruginosa* and severe neutrophilic pulmonary inflammation. *P. aeruginosa* undergoes extensive genetic adaptation to the cystic fibrosis (CF) lung environment, and adaptive mutations in the quorum sensing regulator gene *lasR* commonly arise. We sought to define how mutations in *lasR* alter host-pathogen relationships. We demonstrate that *lasR* mutants induce exaggerated host inflammatory responses in respiratory epithelial cells, with increased accumulation of proinflammatory cytokines and neutrophil recruitment due to the loss of bacterial protease-dependent cytokine degradation. In subacute pulmonary infections, *lasR* mutant-infected mice show greater neutrophilic inflammation and immunopathology compared with wild-type infections. Finally, we observed that CF patients infected with *lasR* mutants have increased plasma interleukin-8 (IL-8), a marker of inflammation. These findings suggest that bacterial adaptive changes may worsen pulmonary inflammation and directly contribute to the pathogenesis and progression of chronic lung disease in CF patients.

## INTRODUCTION

Progressive lung disease is the primary cause of symptoms and early death in patients with the genetic disease cystic fibrosis (CF). In the CF airways, chronic bacterial infections are associated with an exuberant neutrophil-dominant inflammatory response that causes lung damage. Most CF patients are chronically infected with the opportunistic pathogen *Pseudomonas aeruginosa* for decades. Because host defenses fail to clear bacteria, the ongoing interplay between pathogen and host drives inflammation and the immunopathology associated with CF chronic *P. aeruginosa* infections (1, 2).

During its residence within the host, *P. aeruginosa* evolves and genetically adapts to the CF lung environment (3–10). *P. aeruginosa* isolates from chronic infections differ genotypically and phenotypically from those isolated at early stages of infection or from the environment, and commonly display adaptive changes such as conversion to mucoidy or loss of motility. Strikingly, CF-adapted *P. aeruginosa* isolates have reduced expression of acute virulence factors such as pilus, extracellular toxins, and enzymes that cause invasive disease (3, 7–10), suggesting that bacterial factors required for acute virulence are not necessary for chronic infections.

Quorum sensing is a bacterial communication system that allows organisms to coordinate the expression of genes implicated in infection pathogenesis and social microbial behavior in a cell density-dependent manner (11). The *P. aeruginosa* transcriptional factor LasR is one of

the major quorum sensing regulators and controls the expression of several exoproducts and acute virulence factors (12).

*lasR* mutants arise from *P. aeruginosa* populations in both in vitro laboratory conditions (13, 14) and in vivo during human infections (4, 8, 15, 16). At least a third of chronically infected CF patients harbor loss-of-function *lasR* mutants (8, 17, 18), and these patients are associated with worse lung function (17). Given that *lasR* mutants are highly attenuated in models of acute infections (10, 19, 20), this striking paradox raises the possibility that CF-adapted *P. aeruginosa* variants contribute to the progression of CF lung disease through mechanisms distinct from those involved during acute infections. How the adaptive microevolution of *P. aeruginosa* modulates host-pathogen relationships and inflammatory responses remains incompletely understood.

Here, we defined the impact of *P. aeruginosa lasR* mutants on inflammatory responses in vitro, in vivo, and in CF patients. We observed that *lasR* mutants induced an exaggerated neutrophil-dominant hyperinflammatory response, and dissected the mechanism for this pathogen-host interplay. Our findings suggest a mechanism by which CF-adapted *P. aeruginosa lasR* variants amplify the inflammation of CF lung disease, thus potentially accelerating disease progression.

## RESULTS

### Loss-of-function *lasR* mutation attenuates acute virulence in a CF-adapted *P. aeruginosa* “late” isolate

Using a pair of *P. aeruginosa* clonally related longitudinal isolates (21), Smith *et al.* previously examined the earliest (“early”) isolate recovered from a CF patient at 6 months of age, and the late isolate from the same patient at age 8 years (8). Using whole genome sequencing, they demonstrated that the CF-adapted late isolate underwent adaptive genetic evolution during chronic infection. Similar to many CF-adapted *P. aeruginosa* isolates, the late isolate has several loss-of-function mutations resulting in the loss of pilus-mediated twitching motility

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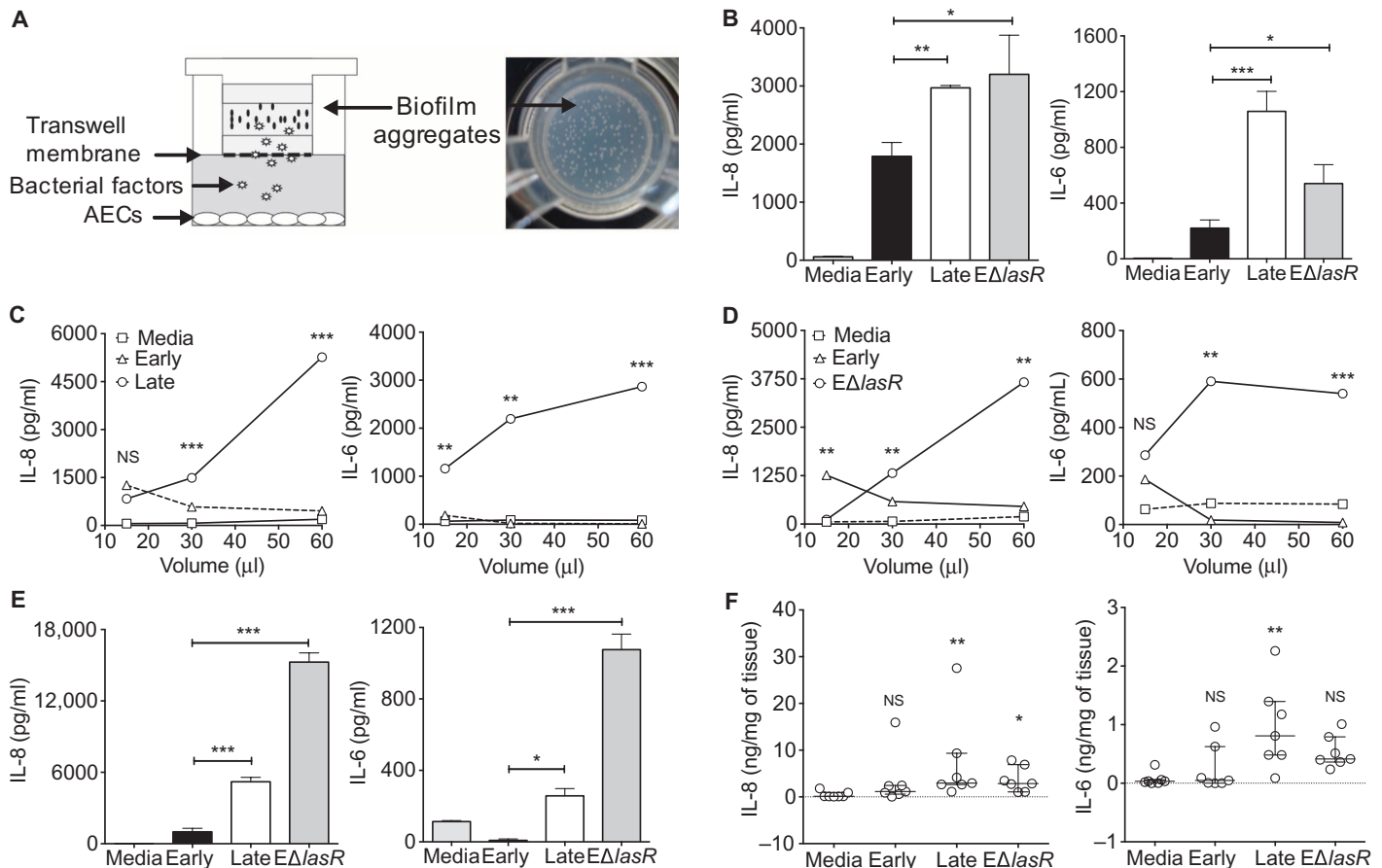
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(*pilA*), pyocyanin (*phzS*), and pyoverdine (*pvdS*) production compared to the early isolate (fig. S1). Notably, the late isolate also carries a loss-of-function nonsense mutation in the *lasR* gene [1–base pair (bp) deletion at position 147], in contrast to a wild-type *lasR* gene sequence in the early isolate (8).

### ***lasR* mutants induce an inflammatory cytokine response in airway epithelial cells**

To begin understanding the consequences of *lasR* mutations on host responses, we first examined the airway inflammatory responses to the early and late pair of isolates. In the CF lung, *P. aeruginosa* grows as biofilm-like aggregates embedded within the mucus layer overlying the airway epithelial surface (22), and the airway epithelium is critical in producing proinflammatory cytokines in response to bacterial stimuli to recruit neutrophils to sites of inflammation (23–26). We therefore used a biofilm–airway epithelial cell (AEC) coculture model where *P. aeruginosa* biofilm aggregates grew in a gel matrix (27) within syn-

thetic CF sputum medium, a defined culture medium that approximates the nutrient composition of CF sputum (28). Human immortalized AECs were cocultured with live biofilms across a permeable membrane (depicted in Fig. 1A), and we measured the major proinflammatory cytokines, interleukin-8 (IL-8) and IL-6, secreted in the AEC culture supernatant (29, 30). Surprisingly, late isolate biofilms were more proinflammatory than were early isolate biofilms: after 24 hours in coculture with *P. aeruginosa* biofilms, secreted IL-8 and IL-6 levels were respectively 1.7-fold ( $P \leq 0.01$ ) and 4.7-fold ( $P \leq 0.001$ ) higher in AECs stimulated with late compared to those stimulated with early isolate biofilms (Fig. 1B). To examine the specific contribution of the *lasR* mutation to this effect, we also tested the  $\Delta$ *lasR* mutant, an early isolate harboring a genetically engineered *lasR* knockout mutation. AECs cocultured with  $\Delta$ *lasR* biofilms also produced IL-8 levels equivalent to those in AECs cocultured with late isolate biofilms and 1.8-fold higher ( $P \leq 0.05$ ) than those in AECs cocultured with early isolate biofilms (Fig. 1B). Similarly, the levels of IL-6 in AECs cocultured with  $\Delta$ *lasR* biofilms were 2.5-fold



**Fig. 1. *lasR* mutants induce a proinflammatory cytokine response in several airway epithelial culture systems.** (A) Schematic and photograph (top view) of a biofilm–AEC coculture system with *P. aeruginosa* biofilm aggregates grown for 48 hours in synthetic cystic fibrosis sputum medium (SCFM) with 0.8% agar in a Transwell permeable support. (B) BEAS-2B cells cocultured with early, late, and  $\Delta$ *lasR* biofilm aggregates (or media control) for 18 hours. (C and D) BEAS-2B cells stimulated with filtrates from early, late, or  $\Delta$ *lasR* isolates for 8 hours at the indicated volumes. (E) CFBE41o- ( $\Delta$ F508/ $\Delta$ F508) cells stimulated with 30  $\mu$ l of early, late, and  $\Delta$ *lasR* filtrates for 18 hours. (F) Ex vivo nasal explants stimulated with 60- $\mu$ l filtrates or media control for 24 hours. IL-6 and IL-8 levels in the AEC culture conditioned supernatants after coculture with biofilm aggregates or stimulation with bacterial filtrates were measured by sandwich enzyme-linked immunosorbent assay (ELISA). Results in (B) to (E) are shown as mean  $\pm$  SEM ( $n \geq 3$  independent biological replicates, representative of  $\geq 2$  independent experiments). Statistical comparisons for (B) to (E) were done using a two-tailed *t* test (versus the early group). Results in (F) are shown as median  $\pm$  interquartile range (IQR) of independent biopsies ( $n = 7$  patients), and statistical comparison was done using the Kruskal-Wallis test (versus control group). \* $P \leq 0.05$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$ . NS, not significant ( $P > 0.05$ ).

( $P \leq 0.05$ ) higher compared to AECs stimulated with early isolate biofilms.

Because both late and  $E\Delta lasR$  isolates induced higher secreted cytokine levels than the early isolate in the biofilm-AEC coculture system, we hypothesized that the cytokine responses were dampened by LasR-dependent extracellular bacterial products because LasR controls the production of many secreted factors. To test this hypothesis, we stimulated AECs with *P. aeruginosa* cell-free filtrates that contained diffusible bacterial products, and again noted that *lasR* mutants (both late and  $E\Delta lasR$  mutants) elicited higher secreted IL-8 and IL-6 levels than did the parental early isolate. As shown in Fig. 1C, the cytokine response to late filtrates was dose-dependent, with IL-8 levels ~3- to 10-fold and IL-6 levels ~6- to 120-fold higher than equivalent stimulation with early filtrates. Results with the  $E\Delta lasR$  mutant filtrates were similar to those of the late isolate (Fig. 1D), indicating that the increased cytokine response was predominantly attributable to *lasR* mutations. Control experiments showed that all *P. aeruginosa* isolates grew to similar bacterial densities in planktonic and biofilm cultures (fig. S2). *P. aeruginosa* cell-free filtrates and live biofilms also caused minimal cytotoxicity (fig. S3, A and B) and loss of viability (fig. S3C) to AECs.

To validate our initial observations with AECs, we tested two additional airway epithelial culture systems. We used CFBE41o- cells, which are a CF AEC line homozygous for the  $\Delta F508$  mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) gene (31) (Fig. 1E), and ex vivo primary human nasal tissues (Fig. 1F) that contain morphologically intact primary upper respiratory tract epithelial cells, as well as stromal structures and immune cells. Stimulation with filtrates from *lasR* mutants induced the highest IL-8 and IL-6 levels in both systems.

### Loss of LasR function causes an exaggerated neutrophil-dominant hyperinflammatory response in vivo

The results from our in vitro experiments demonstrated that *lasR* mutants caused a hyperinflammatory IL-8 and IL-6 cytokine response in AEC. We therefore hypothesized that infection with *lasR* mutants will increase pulmonary recruitment of neutrophils and an exaggerated inflammatory response in vivo compared with the early isolate, which carries a wild-type *lasR* gene. To test this hypothesis, we infected C57BL/6 mice with *P. aeruginosa* embedded in agar beads to create a chronic airway infection model (32, 33). *P. aeruginosa* embedded in agar beads approximate the endoluminal bacterial aggregates observed in chronic CF airway infections by impairing bacterial clearance and create a subacute and nonlethal pulmonary infection. Despite identical bacterial burden at both day 1 and day 4 post-infection (p.i.) (fig. S4A), mice infected with the  $E\Delta lasR$  mutant showed significantly greater neutrophil-dominated pulmonary inflammation than did those infected with the early isolate. At day 4 p.i., the bronchoalveolar lavage fluid (BALF) of  $E\Delta lasR$ -infected mice contained 9.5-fold ( $P \leq 0.01$ ) more leukocytes ( $CD45^+$ ) and 25-fold ( $P \leq 0.01$ ) more neutrophils ( $CD45^+ CD11b^+ Ly6G^{hi}$ ) compared to the BALF of early isolate-infected mice (Fig. 2A). Similarly, we observed 8-fold ( $P \leq 0.001$ ) more leukocytes and 14-fold ( $P \leq 0.001$ ) more neutrophils in the whole-lung homogenates of  $E\Delta lasR$ -infected mice compared to early isolate-infected mice (Fig. 2B). Overall, neutrophils accounted for a greater proportion of leukocytes in  $E\Delta lasR$ -infected compared to early isolate-infected mice (93% versus 27% in the BALF,  $P \leq 0.01$ ; 53% versus 26% in the lung,  $P \leq 0.01$ ) (Fig. 2, C and D). To further characterize the inflammatory cytokine response in vivo, we measured the BALF and plasma cytokine levels at

day 4 p.i. Both BALF levels of KC (keratinocyte-derived cytokine, a murine functional homolog of the human neutrophil chemokine IL-8) and IL-6 were significantly higher in the  $E\Delta lasR$ -infected group, with 8-fold increase for KC ( $P < 0.001$ ) and 10.5-fold increase for IL-6 ( $P < 0.01$ ) compared to the early group (Fig. 3A), consistent with the vigorous neutrophil recruitment to the lung. Blood KC and IL-6 levels also showed similar differences between the early isolate-infected and  $E\Delta lasR$ -infected groups (Fig. 3B).

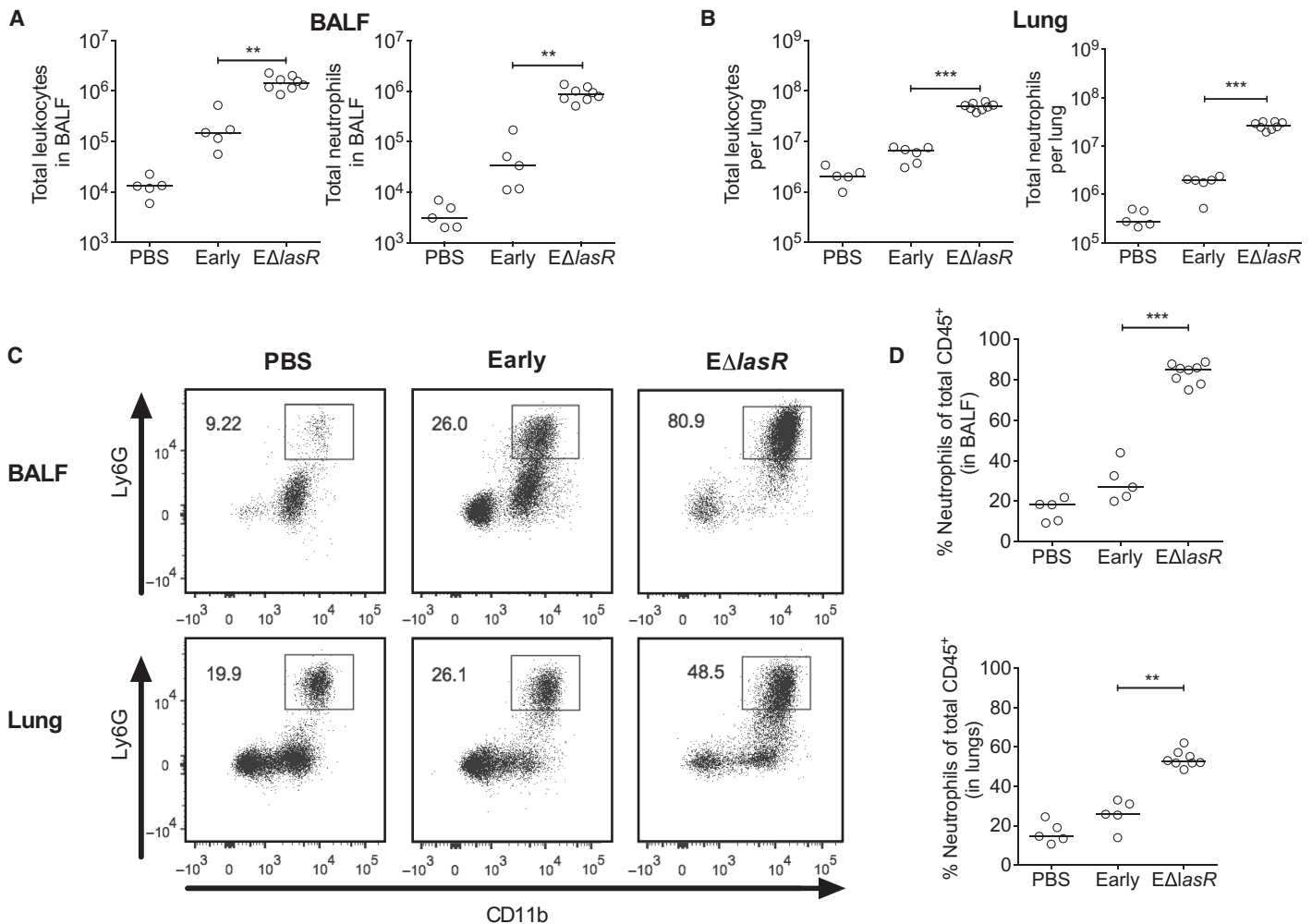
Histology revealed peribronchial polymorphonuclear leukocyte (PMN)-dominant inflammatory foci associated with agar-embedded endoluminal *P. aeruginosa* beads. We also observed scattered areas of PMN-dominant parenchymal inflammation (Fig. 3C). Lungs infected with the  $E\Delta lasR$  mutant, but not the early isolate, displayed rare areas of airway necrosis and alveolar fibrin accumulation, suggesting more pronounced localized tissue damage. We scored the airway and parenchymal inflammation in histological sections of the lung tissue, and only the  $E\Delta lasR$ -infected lungs showed inflammation significantly above the control group (Fig. 3D). We also found higher total protein levels in the BALF, a marker of lung tissue damage, in the  $E\Delta lasR$ -infected mice compared to the early isolate-infected ones (Fig. 3E). Together, these results show that the loss of LasR function in *P. aeruginosa* increases neutrophil recruitment and worsens lung immunopathology in vivo.

### LasR-regulated proteases directly degrade secreted cytokines

Cytokine levels in the conditioned supernatants of AECs stimulated with the early isolate were at times below those in control conditions or nearly undetectable (Fig. 1, D and E), leading us to hypothesize that secreted cytokines were degraded by LasR-controlled factors absent in the late isolate and  $E\Delta lasR$  mutant. To test this hypothesis, we first measured cytokine mRNA expression and secreted cytokine levels simultaneously in AECs stimulated with early and late filtrates in a time-course experiment, and observed that the differences in secreted cytokines could not be attributed to the IL-8 and IL-6 mRNA response (fig. S5). Furthermore, heat treatment of early filtrates restored AEC cytokines to levels greater than those in the late filtrate treatment group (Fig. 4A). These findings suggested that heat-labile bacterial factors significantly dampened secreted cytokine levels, likely through a post-secretion degradation process, and heat inactivation of the early filtrates uncovered the cytokine response to other bacterial factors present in those filtrates.

Because LasR regulates the expression of many extracellular factors, including heat-labile proteases, we next asked whether LasR-controlled proteases directly degraded IL-8 and IL-6. Consistent with our hypothesis, the late isolate and  $E\Delta lasR$  mutant both had nearly undetectable extracellular total protease and elastase activities compared to the early isolate, and both activities in the early isolate were heat-labile (Fig. 4, B and 4C). To confirm that LasR-regulated factors directly degraded IL-6 and IL-8, we incubated recombinant human IL-6 (rhIL-6) and IL-8 (rhIL-8) proteins with *P. aeruginosa* filtrates that contained secreted bacterial products. The early isolate filtrate degraded both cytokines, and this proteolytic degradation activity was abrogated by heat treatment (Fig. 4D). In contrast, both rhIL-6 and rhIL-8 protein levels remained intact when incubated with late or  $E\Delta lasR$  filtrates (Fig. 4D).

Because LasR-dependent regulation of proteases varies among different strains of *P. aeruginosa* (34), we then measured protease production by the laboratory strain PAO1-V (35) and four different CF clinical isolates carrying functional wild-type *lasR* alleles (36) and



**Fig. 2. Loss of LasR function causes a neutrophil-dominant hyperinflammatory response in murine *P. aeruginosa* pulmonary infections.** (A) Number of leukocytes (CD45<sup>+</sup>) and neutrophils (CD45<sup>+</sup> CD11b<sup>+</sup> Ly6G<sup>hi</sup>) in BALF. (B) Number of leukocytes (CD45<sup>+</sup>) and neutrophils (CD45<sup>+</sup> CD11b<sup>+</sup> Ly6G<sup>hi</sup>) in whole lung homogenates. (C) Representative flow cytometry scatter plots of neutrophils (CD45<sup>+</sup> CD11b<sup>+</sup> Ly6G<sup>hi</sup>) in the BALF and whole-lung homogenates. Insets show the proportion of CD11b<sup>+</sup> Ly6G<sup>hi</sup> cells of total live CD45<sup>+</sup> cells. (D) Proportion of neutrophils (CD45<sup>+</sup> CD11b<sup>+</sup> Ly6G<sup>hi</sup>) of all leukocytes (CD45<sup>+</sup>) in BALF and whole lungs. Mice in the PBS (phosphate-buffered saline) control groups are inoculated with sterile PBS agar beads. All results are from samples collected at day 4 p.i. Results in (A), (B), and (D) are shown as median ( $n \geq 5$  mice) of each group. Statistical comparison was done using the Mann-Whitney test as indicated.  $**P \leq 0.01$ ;  $***P \leq 0.001$ .

compared them to their respective genetically engineered *lasR* knock-out mutants. As observed with the early isolate, all of the *P. aeruginosa* isolates displayed extracellular total protease (Fig. 5A) and elastase activities (Fig. 5C) that were abolished by *lasR* mutations. All wild-type parental strains also induced significantly less IL-6 and IL-8 compared with their isogenic *lasR* mutants (Fig. 5D). Because loss of protease activity is commonly seen in CF-adapted *P. aeruginosa* isolates (37, 38), we also examined pairs of clonally related longitudinal *P. aeruginosa* isolates collected from three other CF patients (described in table S1). The CF-adapted isolates L1, L2, and L3 produced less elastase (Fig. 5E) and induced more IL-6 and IL-8 production in AECs compared to their paired early isolates E1, E2, and E3, respectively (Fig. 5F). Together, these results indicate that LasR-dependent proteolytic degradation of cytokines occurs in multiple *P. aeruginosa* isolates, and this is a major mechanism in the hyperinflammatory cytokine response associated with protease-deficient isolates.

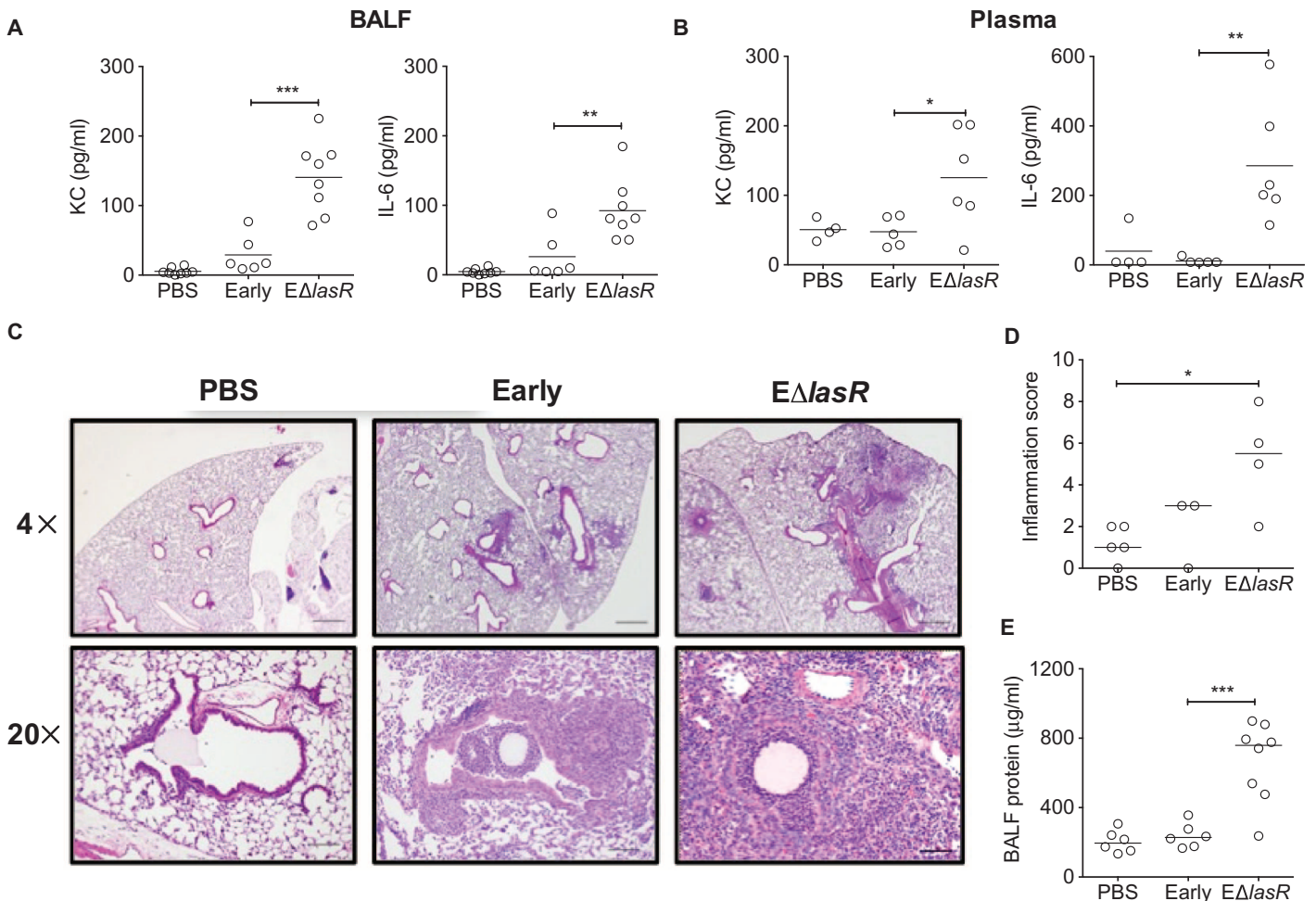
### Bacterial proteases degrade cytokines more efficiently than does neutrophil elastase

Previous studies have reported that neutrophil elastase (NE) can degrade IL-6 and IL-8 (39, 40). Because this enzyme is abundant in the airways of CF patients (41), we compared the cytokine-degrading activities of NE with those of *P. aeruginosa* proteases. At quantities titrated to a specific elastase activity equivalent to purified human NE (2  $\mu\text{g/ml}$ ) (fig. S6), filtrates from *P. aeruginosa* clinical isolates potentially degraded both rhIL-6 and rhIL-8, but NE did not (Fig. 5B). Extracellular bacterial proteases thus likely contribute significantly to cytokine degradation.

### LasB, a LasR-regulated elastase, is required for IL-8 and IL-6 degradation

LasR positively regulates the expression of several *P. aeruginosa* extracellular proteases, including LasA, LasB, and AprA. To determine which LasR-regulated proteases mediate cytokine degradation, we stimulated





**Fig. 3. Greater pulmonary inflammation, systemic inflammation, and lung injury in *EΔlasR*-infected mice.** (A) Cytokine measurement in BALF. (B) Cytokine measurement in plasma obtained from whole blood. (C) Representative hematoxylin and eosin (H&E)-stained lung sections visualized with a 4× (scale bar, 500 μm) or a 20× (scale bar, 100 μm) objective. (D) Total inflammation score. (E) Total protein in BALF. All results are from samples collected at day 4 p.i. Results in (A), (B), and (E) are shown as mean ( $n \geq 4$  mice) of each group, and results in (D) are shown as median ( $n \geq 3$  mice). Statistical comparison was done by two-tailed unpaired Student's *t* test for (A), (B), and (E) and by Mann-Whitney test for (D) as indicated. \* $P \leq 0.05$ ; \*\* $P \leq 0.01$ ; \*\*\* $P \leq 0.001$ .

AECs with culture filtrates from the PAO1-V wild-type strain and its isogenic *aprA*, *lasA*, and *lasB* mutants. The *lasB* mutation had the same effect on cytokine degradation as did *lasR* mutations, whereas deletion of *aprA* and *lasA* had no significant effect (Fig. 6A). To confirm the role of LasB, we deleted *lasB* in the early isolate and complemented the late isolate with an inducible *lasB* gene, thus creating the *EΔlasB* and *L+lasB* strains, respectively. As shown in Fig. 6B, the *EΔlasB* isolate induced a proinflammatory cytokine response, whereas overexpression of *lasB* in the late isolate restored cytokine degradation. Together, our results show that the LasB is necessary and sufficient to degrade IL-6 and IL-8 in AEC cultures.

#### Loss of *P. aeruginosa* LasB activity stimulates neutrophil recruitment

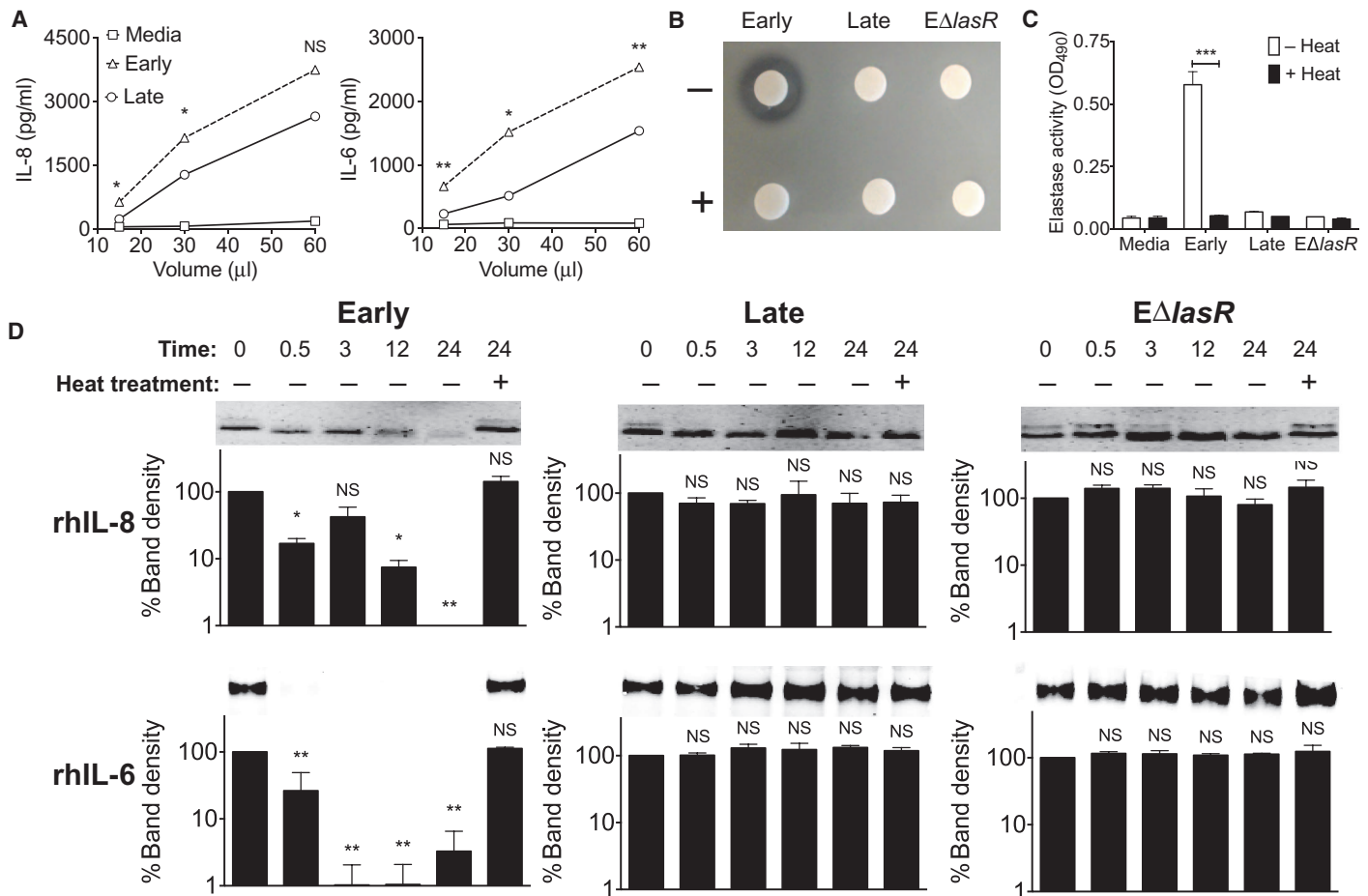
If the LasB-mediated cytokine degradation contributes to the hyperinflammatory responses of *lasR* mutants, we reasoned that the loss of LasR and LasB function should be sufficient to increase neutrophil recruitment. To test this hypothesis, we used a neutrophil transmigration assay to determine the functional consequences of AEC cytokine

response on primary human neutrophil recruitment and chemotaxis in vitro. We collected the conditioned supernatant from AECs stimulated with early, late, *EΔlasR*, and *EΔlasB* filtrates and measured the effects of these supernatants on neutrophil chemotaxis and transmigration.

As shown in Fig. 6C, conditioned supernatant from AECs stimulated with late, *EΔlasR*, and *EΔlasB* filtrates induced 4.5-, 3.5-, and 6.8-fold greater neutrophil transmigration, respectively, compared to AECs stimulated with early filtrates (all  $P < 0.001$ ). These results therefore demonstrate that *P. aeruginosa* mutants lacking LasB function, through either mutation of *lasB* or inactivation of its transcriptional regulator *lasR*, induce greater neutrophil recruitment than do wild-type *P. aeruginosa*.

#### Chronic airway infection with *P. aeruginosa lasR* mutants is associated with higher levels of plasma IL-8 in CF patients

On the basis of our in vitro and in vivo results, we predicted that chronic *lasR* mutant infections would also be associated with a hyperinflammatory response in CF patients. In a prospective cohort of 17 adult CF patients chronically infected with *P. aeruginosa*, we analyzed patients' whole-sputum samples by FREQ-seq analysis (42) to determine the rel-



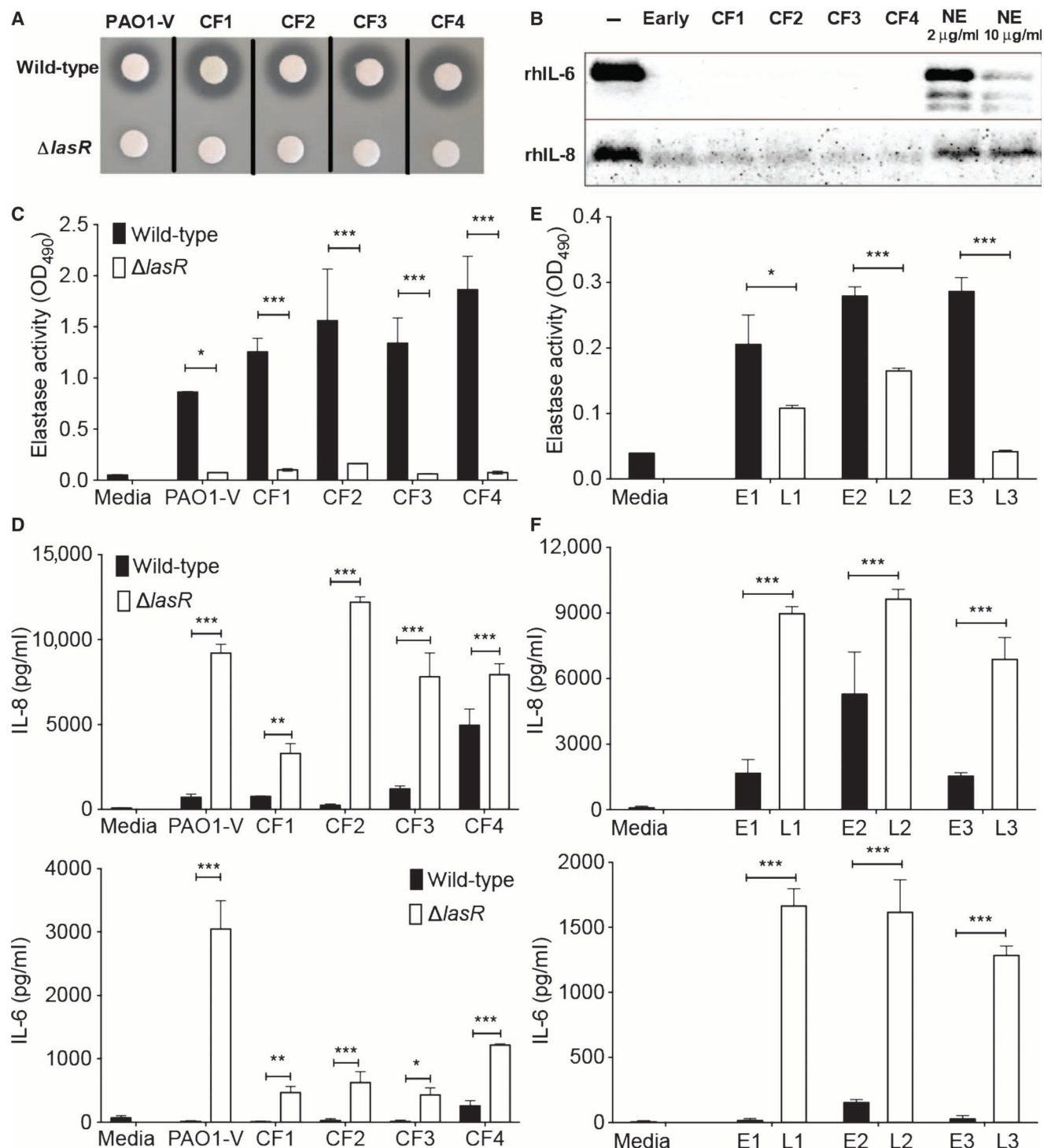
**Fig. 4. *LasR*-regulated proteases directly degrade cytokines.** (A) BEAS-2B cells stimulated with heat-treated early or late filtrates for 8 hours at the indicated volumes. IL-6 and IL-8 levels in the AEC conditioned supernatant after stimulation were measured by sandwich ELISA. (B) Total secreted protease activity in filtrates (+/– heat treatment) on skim milk agar plates. (C) Specific elastolytic activity in filtrates (+/– heat treatment) using the elastin Congo red assay. (D) rhIL-6 or rhIL-8 (10 μg/ml) incubated with filtrates (+/– heat treatment) starting at time 0. The rhIL-6 or rhIL-8 levels were measured by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) followed by Western blot. Bar graphs indicate the relative amounts of protein measured by band densitometry analysis relative to  $T = 0$ . Results in (A), (C), and (D) are shown as mean ( $\pm$ SEM) and are representative of at least three independent experiments. Statistical comparisons were done using a  $t$  test compared with the early group for (A) and (C), and with  $T = 0$  in the same condition for (D). \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

ative frequency of *lasR* mutant alleles, defined by the identification of nonsynonymous mutations within the *lasR* coding sequence. As shown in Fig. 7A and fig. S7A, some patients were colonized with mixed *P. aeruginosa* populations with both wild-type and mutant *lasR* alleles. We defined patients' *lasR* status as *lasR*<sup>mut</sup> if their sputum *P. aeruginosa* populations contained >50% *lasR* mutant alleles and as *lasR*<sup>wt</sup> if they contained <50% *lasR* mutant alleles. On the basis of the frequency of *lasR* mutant alleles in their sputum samples, 8 of 17 patients were considered *lasR*<sup>mut</sup> (Fig. 7A), and the *lasR*<sup>mut</sup> and *lasR*<sup>wt</sup> groups were similar in age (fig. S7B) and gender (female, 50% versus 55% in the *lasR*<sup>mut</sup> and *lasR*<sup>wt</sup> groups, respectively). We concurrently measured the subjects' plasma levels of IL-8, a systemic marker of inflammation in CF lung disease (43, 44). The *lasR*<sup>mut</sup> group had higher IL-8 levels compared to the *lasR*<sup>wt</sup> group during a stable clinical state [median (IQR), 6.2 (4.7 to 18.7) versus 3.4 (2.7 to 5.2),  $P \leq 0.03$ ] (Fig. 7B), and results remained similar even when the patients' *lasR*<sup>mut</sup> status was defined as >20 or >90% *lasR* mutant alleles instead of >50%. Notably, the *lasR*<sup>mut</sup> group was still associated with higher IL-8

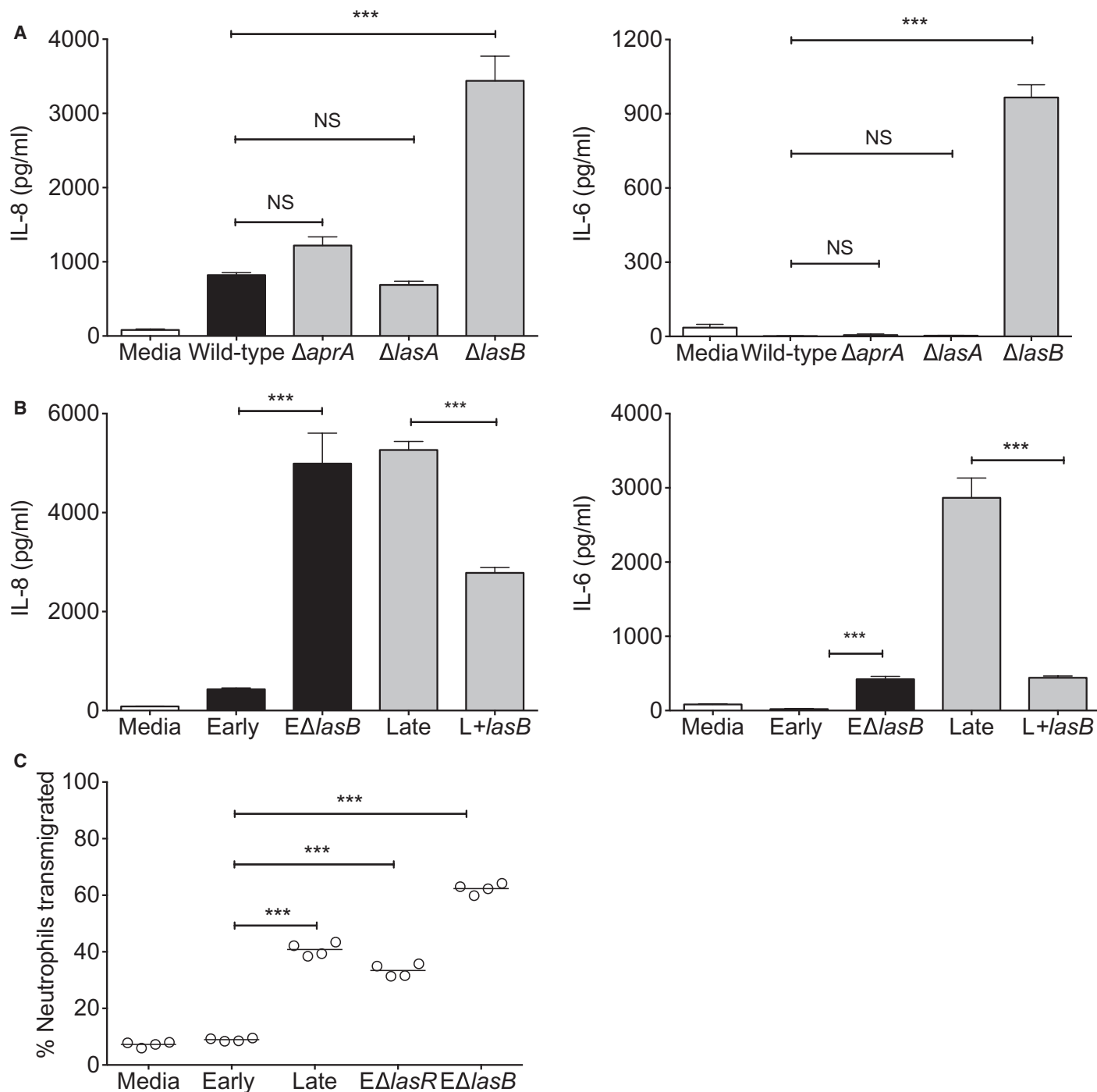
levels when patients were assessed longitudinally [median (IQR), 6.4 (5.0 to 16.0) versus 3.9 (3.2 to 7.1),  $P \leq 0.005$ ] (fig. S7C), with a positive correlation between *lasR* mutant allele frequency and IL-8 levels (Spearman  $r = 0.33$ ; 95% CI, 0.03 to 0.58;  $P \leq 0.05$ ) (Fig. 7C). These results thus show a significant association between airway infections with *lasR* mutants and a marker of inflammation in CF patients.

## DISCUSSION

During the transition from initial to chronic infection, *P. aeruginosa* adapts to the CF lung environment and incurs genotypic and phenotypic changes resulting in reduced expression of invasion and toxicity factors (3, 4, 6–10, 45–49). Although such CF-adapted *P. aeruginosa* mutants should be attenuated in virulence and induce less pathology, in CF patients they are in fact associated with later stages of lung disease that exhibit progressive lung inflammation and dysfunction. To

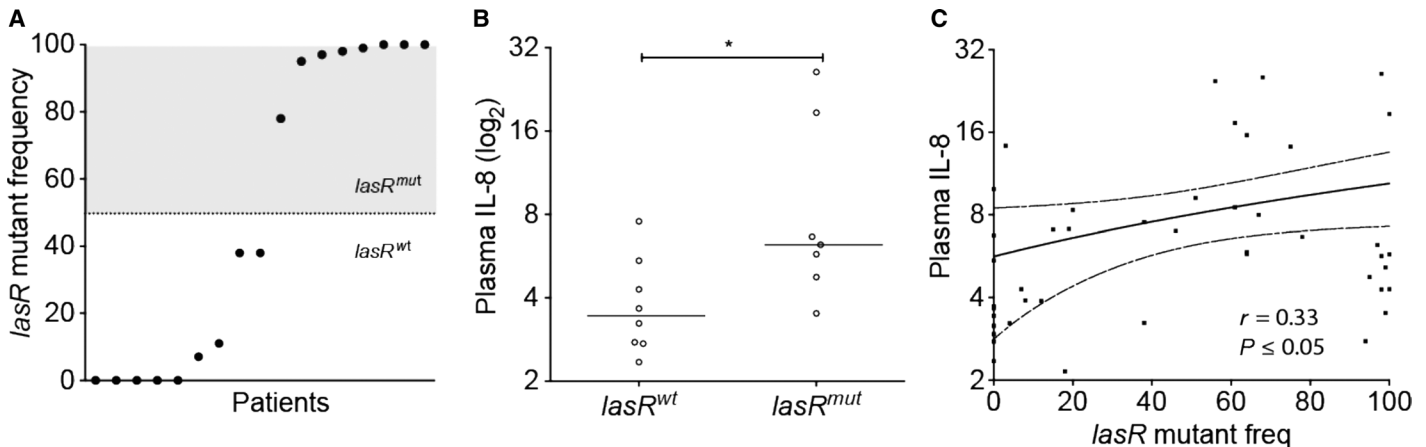


**Fig. 5. *P. aeruginosa* CF clinical isolates degrade cytokines, and protease-deficient isolates induce greater IL-8 and IL-6 response in AEC.** (A) Total secreted protease activity in filtrates from the laboratory strain PAO1-V, clinical isolates (CF1 to CF4), and their isogenic  $\Delta lasR$  mutants assessed on skim milk agar plates. (B) rhIL-6 or rhIL-8 (10  $\mu$ g/ml) incubated with filtrates or purified human NE (2 or 10  $\mu$ g/ml) for 3 hours (rhIL-6) or 24 hours (rhIL-8). CF1 to CF4 filtrates were dosed at equivalent elastase activity as NE (2  $\mu$ g/ml) (as demonstrated in fig. S6). (C) Specific elastase activity in filtrates using the elastin Congo red assay. (D) BEAS-2B cells stimulated with 60- $\mu$ l filtrates or media control for 24 hours. (E) Specific elastase activity in filtrates using the elastin Congo red assay. E1-L1, E2-L2, and E3-L3 are clonally related longitudinal *P. aeruginosa* isolates from three CF patients (as outlined in table S1). (F) BEAS-2B cells stimulated with 60- $\mu$ l filtrates or media control for 24 hours. For (D) and (F), IL-6 and IL-8 levels in the AEC-conditioned supernatant after stimulation were measured by sandwich ELISA. Results in (C) to (F) are shown as mean ( $\pm$ SEM) of  $n = 3$  independent biological replicates and are representative of  $n \geq 2$  independent experiments. Statistical comparisons were done using a two-tailed  $t$  test as indicated. \* $P \leq 0.05$ ; \*\* $P \leq 0.01$ ; \*\*\* $P \leq 0.001$ .



**Fig. 6. Loss of LasR-regulated LasB abrogates IL-6 and IL-8 degradation and induces more neutrophil recruitment in vitro.** (A) BEAS-2B cells stimulated with 60- $\mu$ l filtrates or media control for 8 hours from PAO1-V (wild-type), and its derived  $\Delta aprA$ ,  $\Delta lasA$ , and  $\Delta lasB$  mutants. (B) BEAS-2B cells stimulated with 60- $\mu$ l filtrates or media control for 8 hours from early, E $\Delta lasB$ , late, and L+*lasB* (complemented with *lasB*) isolates. (C) Human neutrophil transmigration assay with conditioned medium from BEAS-2B cells stimulated with 60- $\mu$ l filtrates or media control for 24 hours. For (A) and (B), IL-6 and IL-8 levels in the AEC conditioned supernatants after stimulation were measured by sandwich ELISA. Results are shown as mean ( $\pm$ SEM) of  $n = 3$  independent biological replicates and are representative of at least  $n \geq 3$  independent experiments. Statistical comparisons were done using a two-tailed  $t$  test as indicated. \* $P \leq 0.05$ ; \*\* $P \leq 0.01$ ; \*\*\* $P \leq 0.001$ .





**Fig. 7. Colonization with *lasR* mutant *P. aeruginosa* is associated with higher plasma IL-8 in CF patients.** (A) Frequency of *lasR* mutant alleles measured by FREQ-seq in the 17 CF patients, with each dot representing one patient's sputum sample collected during a clinically stable state. The patients' *lasR* status is defined as *lasR*<sup>wt</sup> or *lasR*<sup>mut</sup>, as shown. (B) Plasma IL-8 levels measured in clinically stable CF patients according to their *lasR*<sup>wt</sup> or *lasR*<sup>mut</sup> status. Results are shown as median (*lasR*<sup>wt</sup> group, *n* = 9 patients; *lasR*<sup>mut</sup> group, *n* = 8 patients). Statistical comparison was done using the Mann-Whitney test compared with the *lasR*<sup>wt</sup> group. \**P* ≤ 0.05. (C) Linear correlation between *lasR* mutant allele frequency and plasma IL-8 levels in longitudinal sampling of CF patients (*n* = 17). Spearman correlation *r* = 0.33; 95% confidence interval (CI), 0.03 to 0.58. *P* ≤ 0.05.

explore this paradox, we focused on *lasR* mutants. Our study showed that *lasR* mutants induce greater inflammation in vitro and in vivo despite reduced expression of acute virulence factors. We demonstrated that LasB, a LasR-controlled protease, degraded cytokines, and loss of LasB-dependent proteolysis was sufficient to cause an exaggerated inflammatory response in vitro under biologically relevant conditions. Notably, the loss of LasR function also induced an exaggerated cytokine response associated with increased pulmonary neutrophilic inflammation and airway tissue damage in murine airway infections. These findings thus support a model wherein adaptive *lasR* mutants directly contribute to exaggerated cycles of infection and inflammation, leading to progression of CF lung disease. Consistent with this model, CF patients predominantly infected with *lasR* mutants had higher plasma IL-8 levels than those predominantly infected with wild-type *P. aeruginosa*, whereby circulating IL-8 levels are markers of inflammation elevated in CF lung disease (43, 44). Whether *lasR* mutants cause worsening of lung function over time in CF patients still needs to be addressed in a longitudinal study.

Airway inflammation in CF lung disease is dominated by neutrophils and directly contributes to pulmonary dysfunction and tissue damage (2, 50–52). *P. aeruginosa* typically grows as biofilm aggregates within the viscous mucus layer overlying the airway epithelial surface and is not invasive in CF chronic infections (22, 53). AECs thus interact with extracellular bacterial products to produce proinflammatory mediators critical for recruitment of neutrophils to the sites of inflammation. These interactions are modeled in our experimental systems, and we observed the hyperinflammatory effects associated with *lasR* mutants across several cell culture systems, including CF and non-CF AECs, immortalized as well as primary human cells, using both genetically engineered and naturally occurring *lasR* mutants. We recognize that our murine infection model overlooks the contribution of host CFTR mutations (which cause CF disease) on host defenses and immunity and is not a model of CF lung disease per se. However, it nonetheless provides a well-established in vivo system to dissect the host-pathogen interactions during chronic infection and their effects on pulmonary immunopathology.

At first glance, our findings contrast with previous studies reporting on the proinflammatory effects of LasR-regulated factors. For example, the LasR-dependent quorum sensing signal 3-oxo-C<sub>12</sub>HSL (3-oxo-dodecanoyl-homoserine lactone) can directly interact with host cells via multiple different mechanisms (54), such as activating NF-κB (nuclear factor κ-light-chain enhancer of activated B cells), MAPK (mitogen-activated protein kinase), and Ca<sup>2+</sup>-dependent signaling pathways, leading to induction of proinflammatory cytokine responses (55–59). Azghani *et al.* (60, 61) also reported that LasB can activate MAPK and NF-κB inflammatory signaling pathways in host cells. In contrast to ours, these studies focus on host intracellular signaling responses and mRNA and intracellular cytokine levels and do not capture the contribution of post-secretion cytokine degradation, the major mechanism investigated in our studies. Our results with heat-inactivated filtrates or protease-deficient mutants are in fact consistent with the notion that additional LasR-regulated factors can induce IL-8 and IL-6 production.

However, the resulting secreted cytokine levels are only elevated in the absence of bacterial protease activity. Because protease-mediated cytokine degradation occurs after cytokines are secreted, this process can prevail over more “upstream” bacteria-host interactions that modulate AEC cytokine expression. LasR controls the expression of extracellular proteases in many *P. aeruginosa* strains (62, 63), including the clinical and laboratory strains tested in this study, and LasB can be detected at levels exceeding 100 μg/mg in the sputum of CF patients (64). In protease-deficient *P. aeruginosa* clinical strains, the loss of protease activity is primarily caused by the loss of LasB elastase production (65, 66).

Although the *P. aeruginosa* elastase LasB is established as an acute virulence factor that degrades host matrix proteins and causes tissue damage (67–69), its dual immunomodulatory role is likely unrecognized because our results show that it degrades cytokines more potently than does human NE. In settings where immune responses fail to clear bacteria, such as in the CF airways, the loss of bacterial elastase activity paradoxically amplifies pulmonary neutrophilia owing to reduced cytokine degradation.

LasR-regulated proteases could modulate host inflammatory responses through multiple pathways. For example, *P. aeruginosa* proteases are capable of degrading in vitro cytokines such as RANTES (regulated on activation, normal T cell expressed and secreted), MCP-1 (monocyte chemotactic protein 1), and ENA-78 (epithelial-derived neutrophil activating peptide 78, or CXCL5) (70–72). We focused on IL-8 and IL-6 because AECs secrete them abundantly in response to *P. aeruginosa* to recruit and activate neutrophils (73–76). Sputum IL-8 levels are higher in CF patients infected with *P. aeruginosa* (77) and correlate with decreased lung function (41, 78, 79). LasB can degrade surfactant proteins A and D, leading to resistance to phagocytosis (80–82), and AprA can degrade flagellin monomers, thus dampening TLR5 (Toll-like receptor 5)–mediated responses (83). These observations add to the proposed notion that *P. aeruginosa* proteases disrupt host inflammatory responses, and their loss in CF-adapted *P. aeruginosa* isolates could promote further inflammation.

In many *P. aeruginosa* strains, the quorum sensing regulator LasR controls the expression of a number of acute virulence determinants, including proteases, pyocyanin, and other exoproducts (84, 85). Accordingly, *lasR* mutants are attenuated in several models of acute virulence such as in invertebrate host models (14, 86) and rodent acute pneumonia (20, 85, 87). That the loss of LasR-regulated acute virulence factors does not prevent *P. aeruginosa* chronic infections supports the notion that CF-adapted mutants contribute to the pathogenesis of chronic infection through pathways and host-pathogen relationships different from those involved in acute infections (6).

A convergent adaptive evolution is consistently observed across different *P. aeruginosa* populations in spite of the vast genetic heterogeneity within and between CF patients (4, 8, 49). Significantly, loss-of-function *lasR* mutants commonly arise during chronic *P. aeruginosa* infections to become a dominant variant or occur as part of a mixed *P. aeruginosa* population in the CF lung (8, 15, 17, 36). In a retrospective study of 30 CF patients, *lasR* mutant isolates were identified in 63% of patients (8). More recently, *lasR* mutant *P. aeruginosa* isolates were isolated in 31% of CF patients, and those patients infected with *lasR* mutants were associated with worse age-specific lung function (17), underscoring the clinical relevance of our study. *lasR* mutants can also emerge during the course of non-CF infections, such as in populations of *P. aeruginosa* colonizing intubated patients on mechanical ventilation (88). Furthermore, in a large prospective study of children with CF, the presence of protease-deficient *P. aeruginosa* isolates was among the best bacterial phenotypic predictors of chronic infection (37). Because many of the protease-deficient CF isolates are *lasR* mutants (36), it follows that the loss of LasR-controlled quorum sensing may also be a predictor of more severe chronic infections. Whether this bacterial adaptation is a marker of more severe CF lung disease or contributes to its progression was not addressed by these observational clinical studies.

Loss-of-function *lasR* mutations emerge under certain laboratory growth conditions and often coexist in mixed *P. aeruginosa* populations, as noted in our study and others (4, 13, 15–17). *lasR* mutants may have a fitness advantage, such as growth on nitrate and aromatic amino acid sources (36, 89), higher resistance to alkaline stress (90), and ability to act as social cheaters in mixed *P. aeruginosa* populations (13, 16, 91, 92). Increased  $\beta$ -lactamase activity may also contribute to their selection under  $\beta$ -lactam antibiotic treatment (36). Despite these observations, it remains unknown why *lasR* mutants emerge so readily in the CF lung.

Our results also raise some concerns regarding the proposed development of inhibitors targeting LasR quorum sensing (93) or bacterial proteases (94) as novel antimicrobial agents against *P. aeruginosa*. In chronic CF infections, such agents could induce greater host inflammation and thus cause more harm. Understanding how bacterial adaptation during specific chronic infections modulates host responses and contributes to lung inflammation is critical in developing antimicrobial therapies tailored to the different types and stages of infection.

## MATERIALS AND METHODS

### Bacterial strains, plasmids, and construction of mutants

The bacterial isolates and plasmids used in this study are listed in tables S2 and S3, respectively. The early (AMT0023-30) and late (AMT0023-34) isolates, recovered from the same CF patient at 6 months and 8 years of age, respectively, are clonally related and previously described (8).

The isolates CF1, CF2, CF3, and CF4 were isolated from four different CF patients (36), and their isogenic *lasR* mutants were genetically engineered (36). The longitudinal isolates E1, E2, and E3 and their respective CF-adapted isolates L1, L2, and L3 were clonally related and were recovered from three different CF patients. The construction of the  $\Delta lasB$  and  $\Delta lasR$  mutants and the inducible *lasB*<sup>+</sup> expression construct is described in detail in the Supplementary Methods.

### Bacterial planktonic and biofilm growth conditions

Bacteria were grown at 37°C in SCFM, a defined culture medium that approximates the nutrient composition of CF sputum (28), unless otherwise specified. Planktonic cultures were grown in liquid medium with shaking at 250 rpm. Biofilms were grown as aggregates embedded in agar as described previously (27), with modifications. Briefly, biofilm cultures were inoculated with overnight planktonic cells diluted in 50% SCFM, 20 mM NaNO<sub>3</sub>, and 0.8% melted agar to a final concentration of 5000 cell/ml. Biofilm cultures were grown in 12-mm Transwell permeable supports (Corning, 0.4- $\mu$ m polyester membrane) statically at 37°C for 48 hours. Antibiotics used for selection were gentamicin and tetracycline (50 and 80  $\mu$ g/ml, respectively, for *P. aeruginosa*; 10 and 10  $\mu$ g/ml, respectively, for *Escherichia coli*).

### *P. aeruginosa* filtrate preparation

Planktonic bacterial cultures were grown in SCFM for 24 hours and then centrifuged at 7200g for 10 min at room temperature. The supernatants were filtered with low-protein binding 0.22- $\mu$ m cellulose acetate filters (Corning), and aliquots of sterile filtrates were stored at –20°C, with no more than two freeze-thaws before use. Where indicated, the filtrates were heat-treated for 10 min at 95°C to inactivate proteases.

### AEC cultures and stimulation with *P. aeruginosa* filtrates

Immortalized wild-type human bronchial epithelial BEAS-2B cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM, Wisent) containing D-glucose (4.5 g/liter) and supplemented with 10% heat-inactivated fetal bovine serum (FBS; Wisent), penicillin (100 U/ml), and streptomycin (100  $\mu$ g/ml) at 37°C with 5% CO<sub>2</sub>. For stimulation,  $2 \times 10^5$  cells were seeded in tissue culture–treated 12-well polystyrene plates (Corning Costar) and grown to confluence before the cells were incubated in starvation medium (DMEM supplemented with 0.5%

heat-inactivated FBS and antibiotics) for 16 hours; the cells were then stimulated with 60  $\mu$ l (or otherwise indicated) of sterile *P. aeruginosa* filtrates or SCFM control medium in 1 ml of fresh starvation medium and incubated at 37°C with 5% CO<sub>2</sub> for the indicated time.

Immortalized CFBE41o- cells (31) were routinely cultured in Eagle's minimum essential medium (EMEM) containing D-glucose (1 g/liter) and supplemented with 10% heat-inactivated FBS (Wisent), penicillin (100 U/ml), and streptomycin (100  $\mu$ g/ml) at 37°C with 5% CO<sub>2</sub>. For stimulation,  $2 \times 10^5$  cells were seeded in tissue culture-treated 12-well polystyrene plates (Corning Costar) coated with human fibronectin (10  $\mu$ g/ml; VWR), bovine collagen I (29  $\mu$ g/ml; BD Biosciences), and bovine serum albumin (BSA; 100  $\mu$ g/ml) in LHC basal medium (Invitrogen) and grown to confluence. Before stimulation, the cells were incubated in starvation medium (EMEM supplemented with 0.5% heat-inactivated FBS and antibiotics) for 16 hours, then stimulated with 30  $\mu$ l of sterile *P. aeruginosa* filtrates or SCFM control medium in 1 ml of fresh starvation medium, and incubated at 37°C with 5% CO<sub>2</sub> for 18 hours. For all experiments, the conditioned AEC supernatants were collected after stimulation and stored at -20°C until further analyses.

### Biofilm-AEC coculture system

Biofilm aggregates were grown for 48 hours as described above. Biofilm-containing Transwells were then inserted into tissue culture wells containing a confluent monolayer of BEAS-2B cells immersed in 1 ml of starvation medium for co-incubation at 37°C with 5% CO<sub>2</sub> for the indicated time. For all experiments, the conditioned AEC culture supernatants were collected after stimulation and stored at -20°C until further analyses.

### Ex vivo human nasal explant stimulation with *P. aeruginosa* filtrates

Nasal explant specimens were obtained from human subjects 18 years or older and diagnosed with chronic rhinosinusitis as previously described (95). All subjects participated voluntarily with signed written informed consent, as approved by the Institutional Review Board of the Centre Hospitalier de l'Université de Montréal (CHUM). Surgical specimens were washed in PBS and cut into pieces [mean weight,  $6.9 \pm 0.64$  (SEM) mg]. Each tissue piece was cultured in a 3:1 mixture of DMEM (Invitrogen) and Hanks' buffer solution (Invitrogen) supplemented with penicillin (100 U/ml) and streptomycin (100  $\mu$ g/ml) at 37°C with 5% CO<sub>2</sub> for 72 hours, with replacement every 24 hours. After 72 hours, the samples were stimulated for 24 hours with 60  $\mu$ l of *P. aeruginosa* filtrates or media control. The conditioned tissue supernatant was collected after stimulation and stored at -20°C until further analyses.

### Cytokine measurements in conditioned AEC supernatants

Conditioned supernatants of AEC cultures or nasal explants were centrifuged at 13,000g to pellet cell debris, diluted (1:10 to 1:100 as required), and used for cytokine quantification by ELISA. The levels of IL-6 and IL-8 were quantified by ELISA (BD OptEIA Human IL-6 and IL-8 ELISA kits, BD Biosciences) according to the manufacturer's instructions. All measurements were done with at least three independent biological replicates.

### Chronic murine *P. aeruginosa* pulmonary infection

The pulmonary murine infection with agar-embedded *P. aeruginosa* was performed as previously done (32). Briefly, planktonic bacterial

cultures were grown to an OD<sub>600</sub> (optical density at 600 nm) of 0.5 in LB broth at 37°C with shaking at 250 rpm. Bacterial cells were pelleted by centrifugation, resuspended in sterile PBS, and mixed 1:1 (v/v) with a solution 2% LB and 3% molten agar. Agar-embedded *P. aeruginosa* beads were generated with continuously stirring heavy mineral oil (Fisher Scientific) as previously described (32), and the bacterial density was determined by standard dilution methods after homogenization of beads. Sterile PBS LB agar beads were used in control experiments.

Specific pathogen-free, male C57BL/6 mice (6 to 9 weeks old) were purchased from Charles River Laboratories. Anesthetized mice were infected with 50  $\mu$ l ( $\sim 5 \times 10^5$  colony-forming units per mouse) of the agar bead suspension using a noninvasive intratracheal inoculation method as previously done (32). Mice were humanely sacrificed at designated time points. Whole blood was collected by cardiac puncture, and lungs were perfused with PBS to reduce blood leukocytes. Broncho-alveolar lavage was performed by injecting and aspirating  $4 \times 500$   $\mu$ l of sterile ice-cold PBS through an intratracheal catheter. After the BAL, the lungs were removed and placed in sterile RPMI 1640 medium (Wisent) containing protease inhibitors. For bacterial counts, whole lungs were homogenized and serially diluted for viable bacteria plate counts. All animal experiments were carried out in accordance with the Canadian Council on Animals Care and with approval from the Animal Care Committee of the Research Institute of the McGill University Health Centre.

### BALF, lung homogenates, and plasma sample preparation and analysis

The BALF was centrifuged to pellet cells, and aliquots of the supernatant were frozen at -80°C until analysis. BALF total protein was measured using a bicinchoninic acid protein assay kit (Pierce). For lung homogenates, perfused lungs were minced, digested with collagenase (150 U/ml; Sigma-Aldrich), and filtered through a 100- $\mu$ m pore size cell strainer (BD). Red blood cells were removed by hypotonic lysis. The remaining cells were pelleted, resuspended in 1 ml of sterile RPMI 1640, and quantified using an automated cell counter (Z1 cell counter, Beckmann-Coulter). Murine plasma was collected from whole blood mixed with 0.5 M EDTA, and aliquots were frozen at -80°C until analysis. Cytokines were quantified in all samples by ELISA (Mouse IL-6 and KC/CXCL8 DuoSet ELISA kits, R&D Systems).

### Flow cytometry of BALF and lung homogenates

Cells ( $2 \times 10^6$ ) from lung single-cell suspensions or cells from 400  $\mu$ l of BALF were stained with Fixable Viability Dye eFluor780 (Affymetrix eBioscience) and then blocked with anti-murine CD16/CD32 (Affymetrix eBioscience). Cells were then surface-stained with eFluor610-conjugated anti-murine CD45 (30-F11, Affymetrix eBioscience), eFluor710-conjugated anti-murine Ly6G (1A8, Affymetrix eBioscience), and V500-conjugated anti-murine CD11b (M1/70, BD). Finally, cells were fixed (Cytotfix, BD) and analyzed using LSR II flow cytometer (BD Biosciences) and FlowJo 10.0.7 software (Tree Star).

### Lung histopathology

Murine lungs were inflated and fixed overnight with 10% buffered formalin phosphate (Fisher Scientific). Paraffin-embedded tissues were sectioned into three 5- $\mu$ m slices that were 50 to 500  $\mu$ m apart. H&E-stained sections were evaluated by a veterinary pathologist for inflammation and pathology in a blinded manner. Images were acquired using an Olympus BX51 microscope fitted with an Olympus DP70

charge-coupled device camera. To assess the lung inflammation, a semiquantitative score was used (see the Supplementary Methods), and the average score of two sections was reported.

### Cytokine degradation assay by immunoblotting

Lyophilized rhIL-6 or rhIL-8 (PeproTech) at 10 µg/ml final concentration was incubated with *P. aeruginosa* filtrates [3% (v/v) final concentration] at 37°C. At specified times, 10-µl aliquots were mixed 1:1 (v/v) in tricine buffer [200 mM tris-HCl, 40% glycerol, 2% SDS, 0.04% Coomassie blue G-250, 2% β-mercaptoethanol (pH 6.8)] and heated to 95°C for 5 min. Samples were then separated by SDS-PAGE with 16.5% tris-tricine gels. After transfer of gels onto nitrocellulose paper, the blots were blocked with 2% BSA and then immunoblotted with polyclonal human IL-6 or IL-8 antibodies (R&D Systems) diluted in PBS (with 0.1% BSA, 0.05% Tween 20). The signal was detected using donkey α-goat secondary antibody conjugated to IRDye 800 infrared fluorophore (Li-Cor Biosciences) in PBS with 0.05% Tween 20. Images of the fluorescently labeled protein bands were captured with the Odyssey imaging system (Li-Cor Biosciences) and analyzed using ImageJ software [1.47v, National Institutes of Health (NIH)]. The protein band density was quantified and normalized to the *T* = 0 control on the same blot.

### Skim milk agar protease activity assay

The total protease activity of the *P. aeruginosa* filtrates was assessed using 1.5% agar plates containing 1.5% skim milk as previously described (96). Briefly, *P. aeruginosa* filtrates were spotted onto sterile 6-mm paper discs placed on the agar surface, and the plates were incubated for 24 hours at 37°C.

### Elastase activity assay

Elastase activity in the *P. aeruginosa* filtrates was measured using the elastin Congo red elastase assay as previously described with minor modifications (97). Filtrates were incubated with ECR solution [elastin Congo red (5 mg/ml; Sigma), 100 mM tris-Cl, 1 mM CaCl<sub>2</sub> (pH 7.5)] at 37°C for 24 hours with shaking at 250 rpm. Samples were then centrifuged at 2200g for 10 min, and absorbance at 490 nm was measured in the supernatant using a microplate reader (Bio-Rad, model 680). All measurements were done with at least three independent biological replicates.

### In vitro PMN transmigration assay

PMNs were isolated from human blood (98), and samples contained >99% neutrophils. Neutrophil transmigration assays were performed as previously described using a modified Boyden chamber (99). Briefly,  $5 \times 10^5$  PMNs were added to the upper compartment of Transwell inserts with 5-µm pore size permeable polycarbonate membrane (Corning). Conditioned supernatants of stimulated AECs were added to the Transwell lower compartment and incubated for 3 hours at 37°C with 5% CO<sub>2</sub>. Cells from both upper and lower compartments were collected and counted using a cell counter (Z1 Coulter, Beckman). The percent neutrophil transmigration was defined as the percentage of neutrophils in the basolateral compartment compared to the total number of neutrophils in both compartments.

### Clinical data and cytokine measurements in CF patients

CF patients from the Adult Cystic Fibrosis Clinic at the Montreal Chest Institute (Montreal, Canada) were enrolled prospectively. Clinical data, plasma sample collection, and cytokine measurements were

previously described (100). Spontaneously expectorated sputum samples were collected and stored at –80°C until *lasR* FREQ-seq analysis. All clinical data and samples were obtained either (i) at baseline during a period of stable disease, defined by the absence of any pulmonary exacerbations requiring antibiotic therapy in the preceding month, or (ii) longitudinally over a period of 18 months during both stable and exacerbation clinical states. Informed written consent was obtained from all subjects, and the study was approved by the Institutional Review Board of the McGill University Health Centre.

### FREQ-seq analysis of *lasR* alleles in CF sputum

Genomic bacterial DNA was extracted from whole sputum using the PowerSoil DNA Isolation Kit (Mo Bio Laboratories). The *lasR* gene was amplified by polymerase chain reaction (PCR) with *lasR*-specific primers *lasR*-F and *lasR*-R (see table S4 for primer sequences). PCR products were purified using the QIAquick PCR purification kit (Qiagen). Standard Illumina Nextera libraries were constructed using individual PCR products according to the manufacturer's guidelines (Illumina Inc.). Paired-end libraries for each sample were sequenced with an Illumina MiSeq to generate 150-bp reads for an average of at least 20,000 reads per base per sample. Sample sequences were aligned against the *lasR* PAO1 reference sequence ([www.pseudomonas.com](http://www.pseudomonas.com)) using the BWA-MEM algorithm (101). All *lasR* polymorphisms identified were present at a frequency of >5% in at least 10,000 reads of that given sample. Nonsynonymous mutations within the *lasR* coding sequence were considered mutant *lasR* alleles, and patients were considered to be in the *lasR*<sup>mut</sup> group if the *lasR* mutant allele frequency was >50% in that sample.

### Statistical analyses

All results are expressed as mean ± SEM or median ± IQR as indicated. Statistical analyses were done using Prism 5 software (GraphPad). Comparisons were performed using an unpaired two-tailed Student's *t* test or the Mann-Whitney nonparametric test as indicated. Comparisons between three or more groups were performed using one-way analysis of variance with Bonferroni's test or Kruskal-Wallis nonparametric test as indicated. A *P* value of ≤0.05 was considered to be statistically significant. \**P* ≤ 0.05, \*\**P* ≤ 0.01, and \*\*\**P* ≤ 0.001.

### Accession numbers

Accession numbers for genes and proteins mentioned in the text (National Center for Biotechnology Information Entrez gene ID number) are as follows: *P. aeruginosa pilA* (878423), *phzS* (881836), *pvdS* (882839), *lasR* (881789), *aprA* (881248), *lasA* (878260), and *lasB* (880368).

## SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at <http://advances.sciencemag.org/cgi/content/full/1/6/e1500199/DC1>

### Materials and Methods

Fig. S1. The late isolate is impaired in the production of acute virulence factors compared to the early isolate.

Fig. S2. The early, late, and EΔ*lasR* isolates grow to similar bacterial density in planktonic and biofilm cultures.

Fig. S3. *P. aeruginosa* cell-free filtrates and biofilms do not cause significant cytotoxicity to AEC cultures.

Fig. S4. The pulmonary bacterial loads are similar in early and EΔ*lasR* isolate-infected mice.

Fig. S5. Relative expression of IL-6 and IL-8 mRNAs in BEAS-2B cells treated with early and late filtrates.

Fig. S6. Elastase activity of filtrates used to degrade rhIL-8 and rhIL-6.



Fig. S7. Colonization with *lasR* mutant *P. aeruginosa* is associated with higher plasma IL-8 in CF patients.

Table S1. Clonally related E and L isolate pairs from CF patients.

Table S2. *P. aeruginosa* isolates and strains.

Table S3. Plasmids.

Table S4. Primers.

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